Production, Purification and Characterization of Bacteriocin Produced by Novel *L. Pentosus* MW857478 for Enhancement of Food Safety and Shelf-Life of Paneer

Verinder virk[1], Garima Verma[2], Chand Ram[3]

[1]Assistant Professor, [2]Research Scholar, Gurukul Kangri University Haridwar,
[3]Principal Scientist, National Dairy Research Institute Karnal

Abstract

This research paper is based upon the production, purification and characterization of bacteriocin by *L.pentosus*MW857478 followed by its enhancement of food safety and shelf life of paneer. In recent year LAB (Lactic acid bacteria) produced bacteriocin attract a great attention of researcher due to their many potential applications. This paper focused on isolation, identification, evaluation of broad spectrum inhibitory activity, production and purification, characterization and evaluation of food safety and shelf life of paneer. Bcateriocin produced by *L. pentosus* showed antagonistic activity against food spoiling pathogens in broad range of Bacteriocin production parameter was optimized with pH 5.5 incubated at 35°C for *L. pentosus*. Bacteriocin was purified by ammonium sulphate precipitation and purified bacteriocin with single band on SDS-PAGE for molecular weight. The purified bacteriocin stable at 2-10 pH and 30-75°C temperatures, suggesting *L. pentosus* a potent candidates for safety and extending shelf life of paneer for 15 days.

***Keywords**: - Antagonistic activity, Bacteriocin, purification, raw milk, pathogens, lactic acid bacteria*

Introduction

Lactobacilli are important microorganism as they recognised as their many potent abilities in food quality as preservation and also have suitability to produce drastic changes in the taste, flavour and texture, also they show broad range of spectrum against pathogenic and spoilage microorganisms. Therefore they found naturally in fermented foods, assumed to be safe without create any health risk of consumers, and designated as GRAS (Generally Recognized as Safe) organisms because of producing various compounds such as organic acids, hydrogen peroxide and bacteriocin.

Corresponding author:
Verinder Virk
Assistant Professor, Dept. of Microbiology
Kanya Gurukul Campus, Gurukul Kangri University,
Haridwar-249407

Bacteriocins are extracellular peptides or proteins that exhibiting bactericidal activity against species and closely related species [11]. Although they may be found in many Gram positive and Gram negative bacteria, those produced by LAB have received particular attention of consumer in recent year due to their potential application in the food industry as natural preservatives. They have been shown very important role in improving microbiological quality and shelf life of many fermented food products and set very excellent examples of bio-preservation. They are mostly heat stable and responsive for protelolytic inactivation so there role as novel food preservatives have received great attention towards the bacteriocin producing lactic acid bacteria species *Lactococcus, Lactobacillus, Streptomyces, Staphylococcus, Bacillus, Pediococcus* and *Carnobacterium* as reported till now [12].

Materials and Methods

Isolation, phenotypic and molecular characterization of *L.pentosus*

L.pentosus was isolated from raw milk samples from cows and goats were collected from different localities of Roorkee, Uttrakhand state of India. Milk sample was utilized for making initial dilution (10^{-1}). Further serial dilution up to 10^{-6}, 1ml of first dilution was transferred into 9 ml of sterile peptone water. 1ml of this dilution was transferred in sterilized Petri-plates and poured then 15 ml melted and cooled de Man Rogosa (MRS) agar and mixed properly and allowed to cool. Plates were incubated at 37°C/48 hours. After incubation, different types of colonies appeared on plate with different morphology such as colour, shape and size were picked with the help of sterilized tooth picks into sterile MRS agar plates. This process was repeated 2-3 times on fresh MRS agar media by incubating at 37°C/24 hours to have pure cultures [16].The purified cultures were preliminary screened for catalase test. The pure culture were further characterized by Gram's staining and, and other biochemical tests. The cultures were characterized based on cell morphology and biochemical tests [13].The selected strains were identified for molecular characterization of lactobacilli isolates was performed by 16SrRNA gene sequencing [4].

Screening of antagonistic activity of bacteriocin producing *L.pentosus*

L.pentosus exert antagonistic activity against indicator microorganisms *viz., E. coli*(ATCC 25922), *B. cereus*(ATCC 14579), *S. aureus*(ATCC 25923) was performed by disc diffusion method. The isolated *L.pentosus* was inoculated in 5ml MRS broth and incubated 37°C/18-24hrs. Cell-free supernatant (CFS) by centrifugation of this culture at 10000×*g* for 10 min at 4°C. To rule out any possibility of antimicrobial activity due to organic acid (H_2O_2), CFS was adjusted to pH 7.0 by adding 1N NaOH. The CFS also treated with catalase to eliminate the inhibitory effect of H_2O_2 produced by lactobacilli isolate. The discs were prepared from Whattman filter paper and autoclaved at 121°C/15 min. Culture free MRS broth disc were used as negative control. The discs were placed on Muller-Hinton agar (MHA) seeded with 18hrs active culture of indicator microorganism. Plates were incubated at 37°C/ 24hrs for clear zone of inhibition around the discs, used to determine bacteriocin activity according to [3].

Production and purification of bacteriocin

24hr old culture of *L. pentosus* was propagated by10% of inoculum on MRS broth and incubated for 48h at 120 rpm at 37°C. The whole broth centrifuged for 1180g for 15 min and CFS used as crude bacteriocin [11]. The bacteriocin sample protein concentration determined by [8].and bovine serum albumin (BSA) used as standard. For purification of CFS of bacteriocinwas saturated with 60-70% ammonium sulphate and stored at 4°C to precipitate out the proteins, pellets were collected after centrifugation at 1180g at 4°C for 30 minutes.

Molecular weight Determination

The molecular weight of purified bacteriocin was detected by SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis [6].

Characterization of bacteriocin activity on the basis of effect of enzymes, pH, temperature

The bacteriocin of isolate *L. pentosus* showing clear zone of inhibition was characterized for temperature, pH and enzymes [2].

Enzyme(proteinase K, trpsin or pepsin) was tested on the antagonistic activity of crude bacteriocin preparation at a final concentration of 1mg/ml and incubated for 2hr/30ºC,wheras effect of temperature (heat resistance) was tested at different temperatures 30ºC, 45ºC, 60ºC, 75ºC and 100ºC, bacteriocin activity was detected against selected pathogenic bacteria for 30, 45 minutes, Effect of pH on bacteriocin activity was tested at various pH 2.0 to 10.0 adjusting through sterile 1mol/lNaOH or 1 mol/lHCl.

Shelf Life studies of paneer

Preparation of samples

Freshly prepared paneer sample that was prepared in Kanya Gurukul campus, department of microbiology lab the pieces 5gm 3cm×3cm and 0.5mm thickness both the samples was sprayed equally by partially purified bacteriocin 5µg/g on the surface of the piece with the help of hand operated sterile spray bottles and kept and stored at under refrigerated conditions (4°C) and observe at every 3 days interval until spoilage for the parameter via microbiological analysis test like total plate count (TPC), coliform count [1].

Microbiological analysis

The paneer samples were taken out at different time intervals until spoilage. For plate count of total aerobic bacteria, serial dilutions were prepared with normal saline (0.85% NaCl). 1 g of sample was taken and added into 9ml of normal saline solution and termed as 10^{-1} dilution. After mixing it homogenously, 1 ml of sample was taken from 10^{-1} dilution and added in 9 mL of normal saline solution (10^{-2} dilution). Similarly, further dilutions were prepared. From appropriate dilution, 0.1 mL sample was taken and plated on solidified and dried (one day at 37°C) standard plate count agar.Plates were incubated at 37°/48hr and colonies were counted through Darkfield Quebec Colony counter.

Results

Identification of bacteriocin producing LAB

Out of 56 isolates, a total of 18 isolates were analysed for the potential of bacteriocin producing lactobacilli from different milk samples whereas on the basis of morphological, and biochemical characterization of Cm12 were confirmed to be lactobacilli. The data has been summarising in Table 1 and figures 1 and 2.

Table 1: Morphological and biochemical characterization of *L.pentosus*Cm12

S No	Test performed	*L. pentosus*
1	Gram's Reaction	+
2	Shape	Rods
3	Catalase	+
4	Glucose	+
5	Arbinose	+
6	Lactose	+
7	Galactose	+
8	Maltose	+
9	Ribose	+
10	Manitol	-
11	Gas formation	+

(+) Positive test, (-) Negative test

Molecular characterization and submission of sequence to NCBI

Lactobacillus isolates Cm12 was identified by 16S rRNA sequence homology as a strain of *Lactobacillus pentosus.* The 16S rRNA sequence of the isolate Cm12 showed 99.64% identity to *Lactobacillus pentosus* strain LMEM1001. The phylogenetic tree for the isolate was constructed and has been depicted in figure 3. The 16S rRNA gene of isolate Cm12 was successfully sequenced and deposited to gene bank with accession number MW857478 was obtained. BLAST homology search showed 100% sequence similarity with *Lactobacillus pentosus* strain JCM 1149.

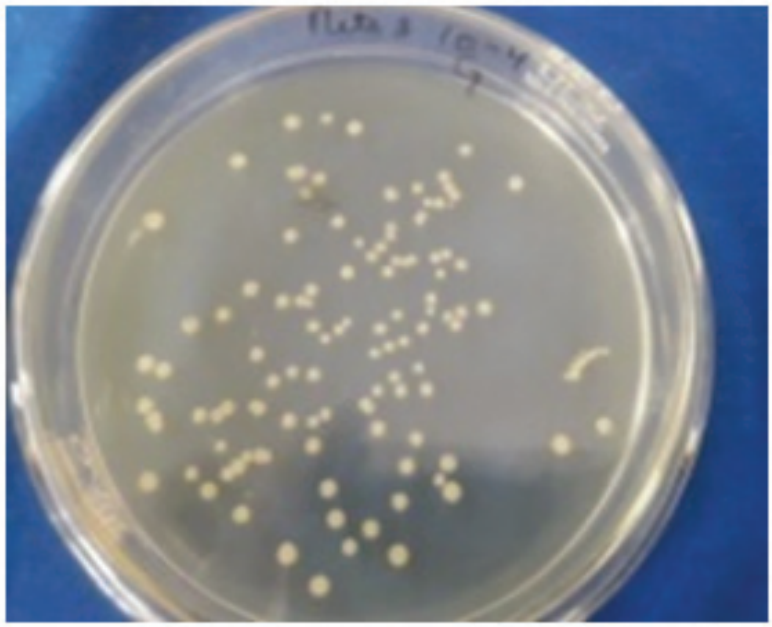

Fig 1: Colony morphology of *L. pentosus* Cm12 on MRS agar.

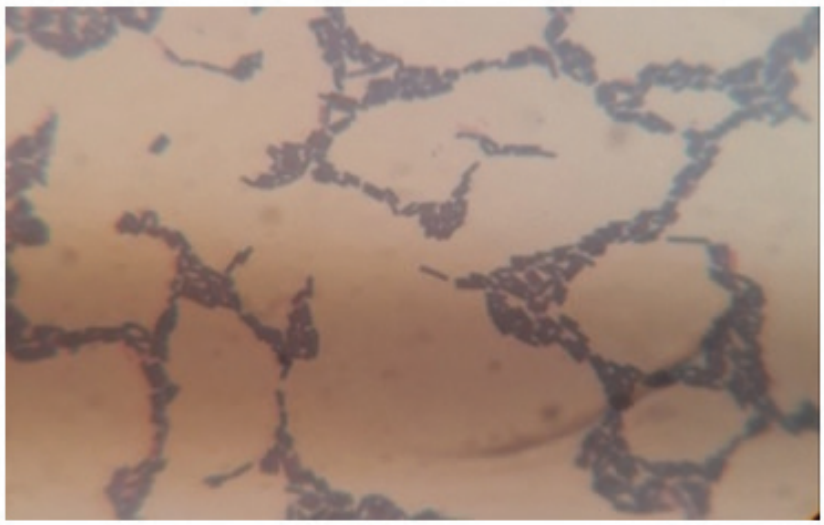

Fig 2: Microscopic observation (1000x) of *Lactobacillus pentosus*

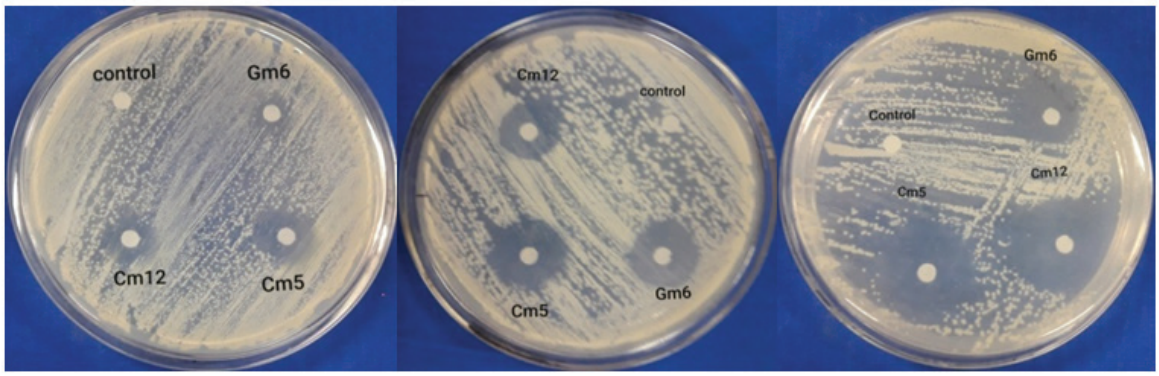

Fig 3: Antagonistic activity against selected food borne pathogens (A) *B. cereus*ATCC14579 (B) *S. aureus*ATCC 25923 (C) *E. coli* ATCC 25922

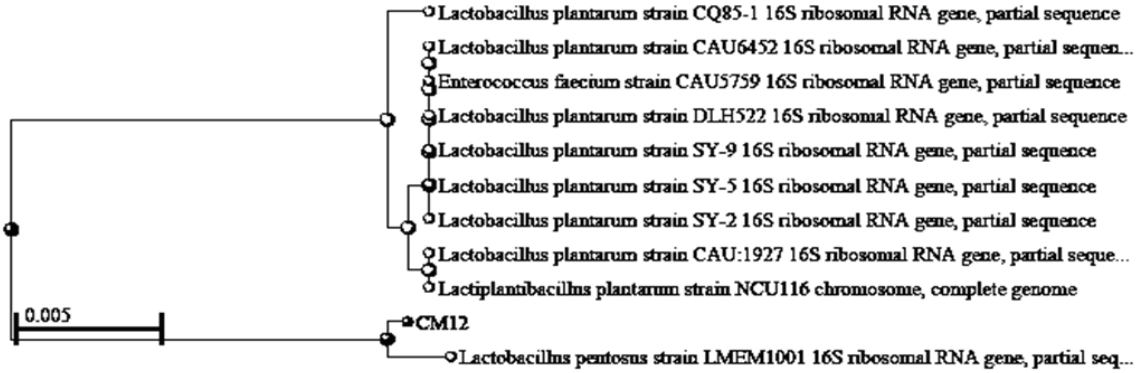

Fig 4: Phylogenetic tree of *L. pentosus* Cm12

Antagonistic activity of bacteriocin producing LAB

Observations on antagonistic activity of identified lactobacilli isolates obtained from cow and goat milk have been presented in table 2 and figure 3. Antagonistic activity of lactobacilli strains was determined by disc diffusion method. The cell free extract was neutralized with 1M NaOH to eliminate acid effect that could inhibit growth of indicator bacteria. *L. pentosus* Cm12 out of these demonstrated highest antagonistic activity i.e. >15 mm zone of inhibition against three tested indicator *S.aureus*ATCC25923,*B.cereus*ATCC14579 and *E. coli* ATCC25922 indicative of broad spectrum inhibition. Our observations are in corroboration also showing inhibitory effect of LAB against *S.aureus* and *B.cereus* to the tune of 8 and 9 mm zone of inhibition [16]. Reported that inhibitory activity of *Lactobacillus* can be enhanced selectively and confirmed that *L.cornyimis* XN8 exhibited broad spectrum antimicrobial effect against *S.aureus*.

Table 2: Antimicrobial activity of bacteriocin producing lactobacilli isolates by disc diffusion method on Muller-Hinton agar.

Isolates	Zone of inhibition (mm)		
	E. coli ATCC25922	B.cereusATCC14579	S.aureusATCC25923
L.pentosus	20.66±0.33	15.33±0.33	18±0.33

*Value of mean of triplicate assays ± standard error, - No zone of inhibition.

Growth and bacteriocin production of *L.pentosus*

The result of the study of growth and bacteriocin production revealed that there was a positive correlation existed between the growth and bacteriocin production.The growth increase was up to 24hour of incubation but bacteriocin production attained maximum at 18hour of incubation and thereafter no increase was noticed maximum bacteriocin production of *L. pentosus* is 6.14 mg/ml. results are summarised in table 3.

Table3: Total protein concentration of bacteriocin produced by *L. pentosus*

Strain	Purification stage	Volume (ml)	Protein concentration (mg/ml)
L. pentosus	Culture supernatant (Crude)	500	21.42
	Ammonium sulphate precipitation (Partial purification)	20	6.14

Determination of molecular weight by SDS PAGE

Molecular weight of *L. pentosus* bacteriocin was carried out by SDS-PAGE (Sodium dodecyl sulphate polyacrylamide gel electrophoresis).Single protein band having molecular weight ±11kDa after stained with coomasie brilliant blue that clearly indicated the purity of protein.

Characterization of bacteriocin

L. pentosus was stable over at 30-75°C and more at 60°C for 30 minutes and declined afterwards against different food borne pathogens (*S.aureus* ,*E.coli* and *B.cerus*) results were showing in figure 5,However pH on bacteriocin activity was tested by incubating at various pH at 2.0-10.0 and the stability of *L.pentosus* on bacteriocin activity is stable at 2.0-10.0 pH showing in Figure 4 and more at 6.0 pH, Whereas *L. pentosus* exhibited complete in inactivation of antimicrobial activity. After the treatment of bacteriocin with proteinase K, trypsin and pepsin which confirms its proteinaceous nature showing in Table 4.

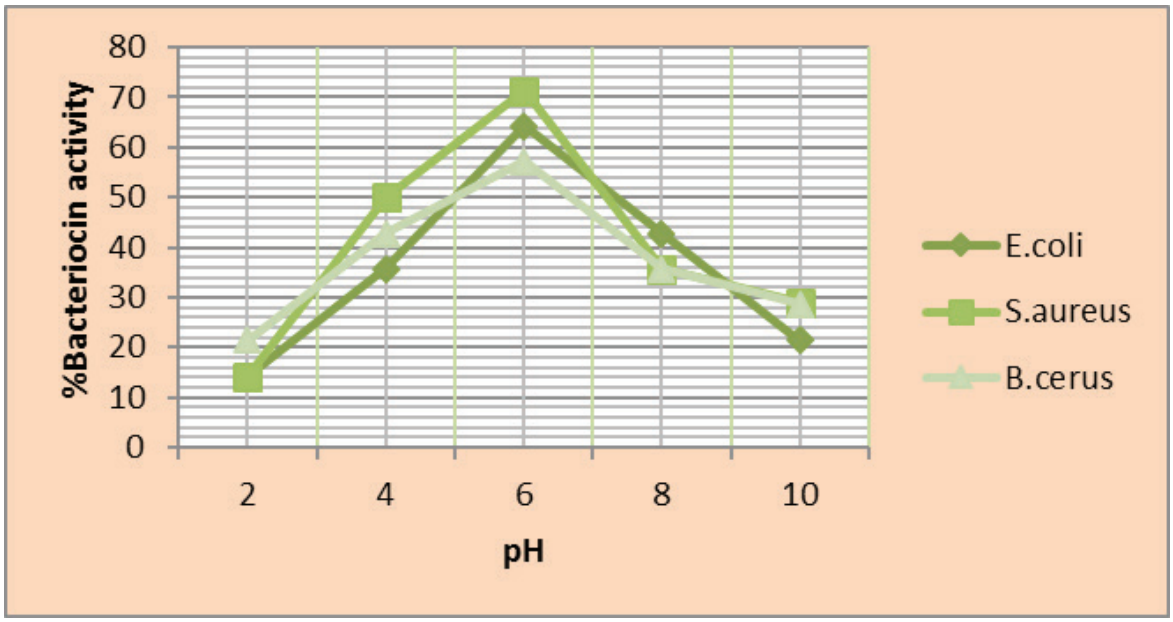

Fig4: Effect of pH on characterization of bacteriocin of *L.pentosus*

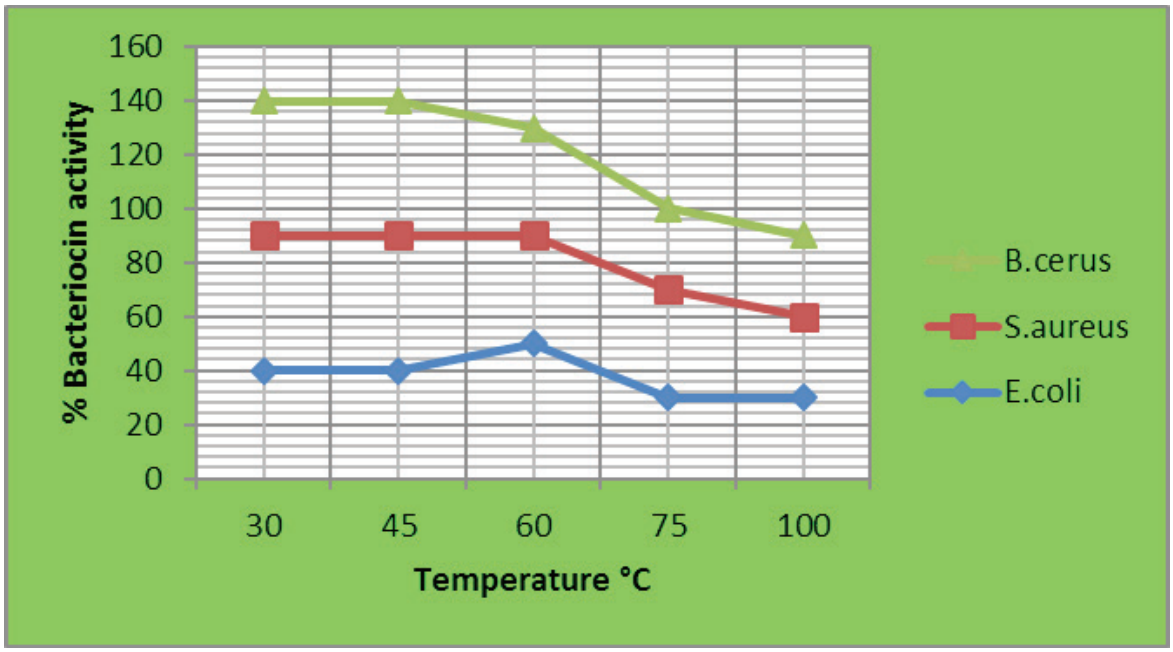

Fig5: Effect of temperature on characterization of bacteriocin of *L.pentosus*

Table4: Showing effect of enzymes on *L. pentosus*

Enzymes	Bacteriocin Activity
	L.pentosus
Protinase K	-
Trypsin	-
Pepsin	-
Catalase	+

Evaluation of Shelf Life Extension Potential of Bacteriocin

Freshly panner sample was prepared in Kanya Gurukul campus, department of microbiology lab the pieces 5gm, 3cm×3cm and 0.5mm thickness both the samples was sprayed equally by partially purified bacteriocin 5µg/g on the surface of the piece with the help of hand operated sterile spray bottles and kept and stored at under refrigerated conditions (4 °C) and observe at every 3 days interval until spoilage for the parameter via microbiological analysis test like total plate count (TPC), coliform count [1] .through microbiological analysis the plates for standard plate count were incubated at 37 °C for 48 h and colonies were counted with the help of Quebec Colony counter. Showing the microbial analysis of paneer for control and pentocin during refrigerator storage (4±1°C) in which there is no coliform detected until day 15 in pentocin while TPC is 4.74±0.08cfu/g at day 15 ofpentocinwhile control TPC at day 3 is 5.82±0.08 cfu/g and not performed (NP), As per the Bureau of Indian standards (IS:1983), the TPC should not exceed 5×10^5 at day 6,9,12 and 15 because numbers are too high for count in control, Hence pentocin bacteriocin could be easily used as biopresevative for extending the shelf life of paneer after incorporated maximum 15days in refrigerator condition (4±1°C).

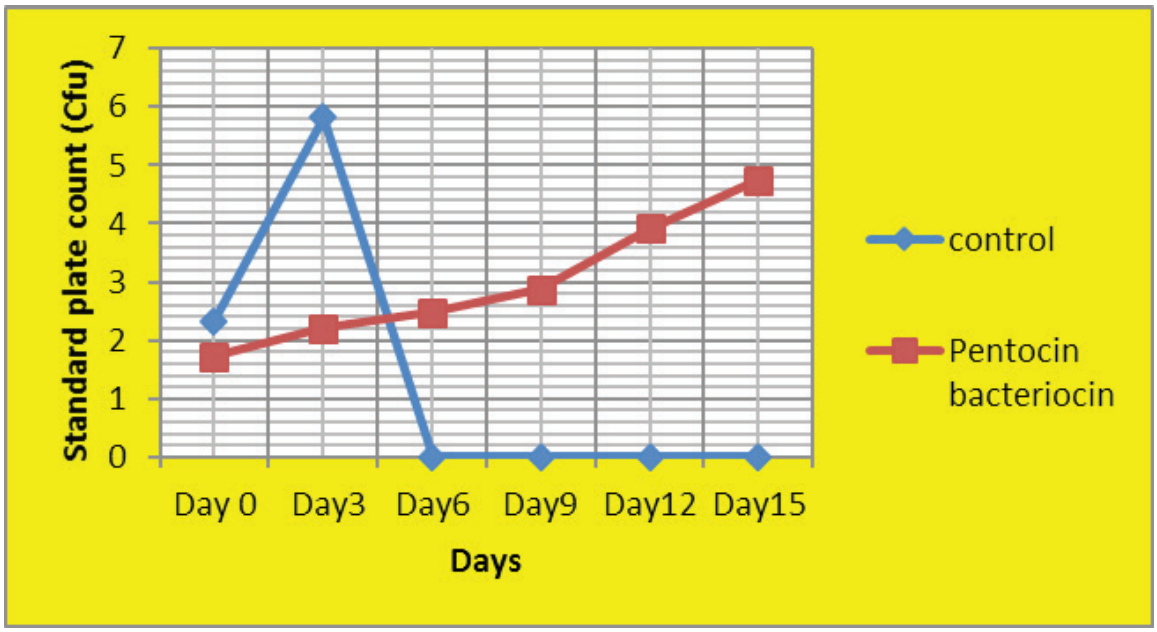

Fig6: Microbiological analysis of paneer with different treatments during refrigerated storage (4±1°C) until spoilage

Discussion

This study aimed to evaluate the ability of *L. pentosus* to produce bacteriocin and enhance the shelf life of paneer. The bacteriocin production and detection was formed in-vitro accompanied by the production of other metabolites like hydrogen peroxide and lactic acid. Thus neutralize the effect of other metabolites and assure that protein extract was not related to these metabolites. The results suggest that a protineceous nature of bacteriocin produced by *L. pentosus*. Based on the described results we conclude that *L.pentosus* produce bacteriocin with an expected size ±11kDa. This molecular weight is within the range of the most frequently reported in [5].Further studied may include the enhancement of shelf-life of paneer.

In parallel we conducted antimicrobial tests of crude and partially purified bacteriocin against different food borne pathogens showing the effectiveness of bacteriocin

against gram negative and gram positive bacteria, then pentocin bacteriocin produced by *L. pentosus* showing the effectiveness in enhancing the shelf life of paneer by incorporating of pentocin.

Acknowledgements

The corresponding author is grateful to Department of microbiology, Kanya Gurukul Campus, Gurukul kangri deemed to be university for providing facilities for research.

Conflict of Interest: The author declares no conflict of interest.

Funding: None.

Ethics Statement: This article does not contain any studies with human participants or animals performed by any of the authors.

References

1. Das A, Chauhan G, Agrawal R K, Tomar S, Sirajuddin S K. Comparative study on evaluation of refrigerated (4±1 C) storage stability of paneer incorporated with crude extract from Indian curd, Nisin and lactic acid. Int J Curr Microbiol Appl Sci. 2018; *7*, 167-80.
2. Elayaraja S, Annamalai N, Mayavu P, Balasubramanian T. Production, purification and characterization of bacteriocin from *Lactobacillus murinus* AU06 and its broad antibacterial spectrum. Asian Pacific journal of tropical biomedicine. 2014: 4: S305-S311.
3. Fossi B, Goghomu S, Tongwa M, Ndjouenkeu R, Cho-Ngwa F. Screening for bacteriocins producing probiotic bacteria from fermented sap of palm trees (Elaeisguineesis and Raffia sudanica): Production and partial characterization of bacteriocins. Journal of Applied Biotechnology and Bioengineering. 2017;*2;*17.
4. Green M R., Hughes H, Sambrook J, Mac Callum P. Molecular cloning: a laboratory manual. In Molecular cloning: a laboratory manual. 2012 ; 1890-1890.
5. Jatmiko YD, Suharto AR, Mustafa I, Aditya, S. Bacterial Population Dynamics of Natural Fermentation of Sumbawa Mare's Milk Using Metagenomic Approach. Microbiology Indonesia. 2021; *15*(2); 2-2.
6. KP S, PV K, PK B, RC R, PB D, AK S. Production and characterization of bacteriocin produced by *Lactobacillus viridescence* (NICM 2167). Brazilian Archives of Biology and Technology 2016; *59*.
7. Laemmli U K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970; *227*(5259); 680-685.
8. Lowry OH, Resebrough NJ, Farr AL, Randall R.J. Protein measurment with folin phenol reagent. Journal of Biological Chemistry. 1951; 93; 265-275.
9. Marchesi JR, Sato T, Weightman AJ, Martin TA, Fry JC, Hiom S J, Wade WG.. Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. Applied and environmental microbiology. 1998; 64(2);795-799.
10. Marmur J, Doty P. Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. Journal of molecular biology1962; *5*(1), 109-118.
11. Ogunbanwo S T, Sanni A I, Onilude A A. Characterization of bacteriocin produced by *Lactobacillus plantarum* F1 and Lactobacillus brevis OG1. African Journal of Biotechnology.2003; *2*(8);219-227.
12. Pato U, Riftyan E, Ayu D F, Jonnaidi N N, Wahyuni M S, Feruni J A, Abdel-wahhab M A. Antibacterial efficacy of lactic acid bacteria and bacteriocin isolated from Dadih's against *Staphylococcus aureus*. Food Science and Technology.2021.
13. Rendtorff E C, Holt C J. The Experimental Transmission of Human Intestinal Protozoan

Parasites. IV. Attempts To Transmit Endamoeba Coli And Giardia Lamblia Cysts By Water. American Journal of Hygiene.1954; 60(3); 32738.

14. Sieladie DV, Zambou N F. Katcham PM, Cresci A, Fonteh F. Probiotic Properties Of *Lactobacilli* Strains Isolated From Raw Cow Mil In The Western Highlands Of Cameroon. Innovative Romanian Food Biotechnology.2011; 9; 12.
15. Vantsawa P A, Maryah UT, Bulus T. Isolation and Identification Of Lactic Acid Bacteria With Probiotic Potential From Fermented Cow Milk (Nono) In Unguwarrimi Kaduna State Nigeria. American Journal of Molecular Biology.2017; 7(2); 99-106.
16. Yuguo Z, Wei Z, Lanhui W, Xiaohu W, Xue C. Effect of Different Levels Of Calcium Diets On Serum Calcium Levels And Calcium Metabolism In The Peripartum Dairy Cows. Feed Industry.2016;10.